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13. ABSTRACT (Maximum 200 words) <p>The role of the RAK tyrosine kinase in breast cancer is unknown at this time. Over the past year efforts in our lab have been made to determine the level of expression of RAK mRNA in cultured cell lines. Two techniques were used to address this question. The first technique involved the use of S1 nuclease protection assays. It was concluded that the probes used in the assay were of too low a specific activity to accurately detect and quantify RAK mRNA. The second technique involved using an electrochemical biosensor that is based upon the detection of guanine base oxidation in target nucleic acid sequences. While initial results with this biosensor seemed to indicate that RAK mRNA was detected several theoretical considerations call this data into question.</p> <p>We still believe that it is possible to quantify the level of RAK mRNA expression in both cultured cell lines and in tumors samples, and we are pursuing different approaches to address the question. We have begun designing a competitive RT-PCR procedure that should be sensitive enough to quantify RAK mRNA expression, and we are in the process of modifying our electrochemical biosensor to increase its sensitivity dramatically.</p>				
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FOREWORD

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Paul Brinksted 7/28/98
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INTRODUCTION

In recent years there has been a dramatic increase in the understanding of the molecular and genetic basis for cancer¹. Of the many genes shown to be either transforming or tumor suppressing in function, a significant portion are tyrosine kinases. Many of these genes have been studied extensively, and some have proven to be useful prognostic markers in evaluating patients with breast cancer.

Research by Slamon et al. revealed that overexpression of the gene encoding the tyrosine kinase HER-2 (erb-2) correlates with reduced long term survival in women with node positive breast cancer². A fluorescence in situ hybridization (FISH) based test is now available to determine the HER-2 status of surgically removed tumors³. Similar results have been found by Cance et al. in their studies on the Focal Adhesion Kinase (FAK). FAK overexpression has been shown to correlate with increased metastatic or invasive potential in breast, colon, and thyroid tumors⁴. One can envision a bioassay similar to the HER-2 assay that would allow clinicians to determine the FAK status of a tumor.

A recently discovered tyrosine kinase, RAK, is thought to play a role in the carcinogenesis of some tumors; however, RAK's structure and biological function are poorly understood. It is believed that RAK mode of action involves binding to Cdc2 through an SH-3 domain (amino acids 86-97)⁵.

The goal of this research is to study RAK mRNA expression in tumor samples and cancer cell lines. Once RAK expression is determined in cell lines, further experiments will be performed to monitor Cdc2 and RAK mRNA expression in cell lines that are microinjected with RAK 86-97 peptide. mRNA expression is going to be determined through various techniques including nuclease protection assays, competitive RT-PCR, and electrochemical biosensors that are being developed in our lab.

The electrochemical biosensor that is being developed in our lab is based upon the catalytic oxidation of the nucleobase guanine. Of the five common nitrogenous bases found in RNA and DNA, guanine has the lowest oxidation potential ($E^0 = 1.05 \text{ V vs.}$

Ag/AgCl)⁶. Previous work conducted in the Thorp lab has shown that the inorganic metal complex $\text{Ru}(\text{bpy})_3^{2+/3+}$ (bpy = 2,2' bipyridine) can serve as an "electron shuttle" to mediate the transfer of an electron from guanine to an electrode surface⁷. Cyclic voltammetry of $\text{Ru}(\text{bpy})_3^{2+/3+}$ in aqueous solution shows that the $\text{Ru}(\text{bpy})_3^{2+/3+}$ couple exhibits a quasi-reversible wave at $E^0 = 1.06 \text{ V}$ vs. Ag/AgCl. When cyclic voltammetry is performed on a solution of $\text{Ru}(\text{bpy})_3^{2+/3+}$ in the presence of guanine nucleotides, an enhancement is

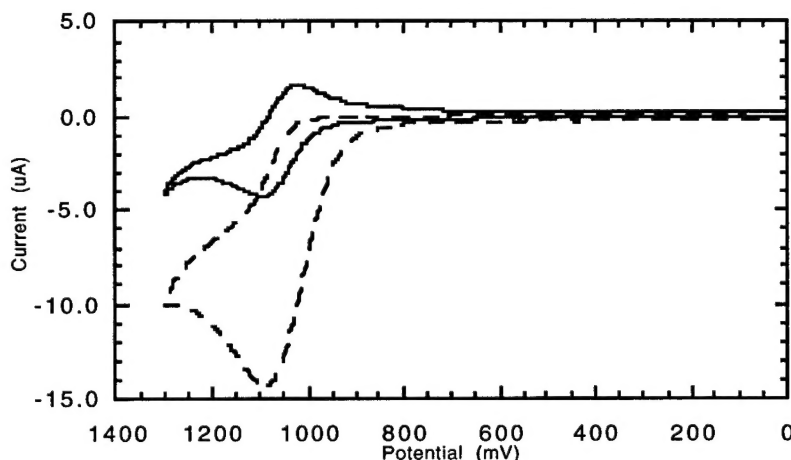


Figure 1: Cyclic voltammograms of $100 \mu\text{M}$ $\text{Ru}(\text{bpy})_3$ in phosphate buffer (line) and in the presence of 3.0 mM herring testes DNA (dashed). CV's were taken at 25 mV/s with an Ag/AgCl reference electrode.

observed in the oxidation of $\text{Ru}(\text{bpy})_3^{2+}$ to $\text{Ru}(\text{bpy})_3^{3+}$ (Fig. 1). This enhancement is observed because upon oxidation, $\text{Ru}(\text{bpy})_3^{3+}$ is reduced by the guanine bases in solution through an outer sphere mechanism, allowing the $\text{Ru}(\text{bpy})_3^{2+}$ to be re-oxidized at the electrode surface several times during the course of the cyclic voltammogram⁷.

Previous work, also performed in our lab, has revealed that specific DNA sequences can be detected via the same electrochemical system through hybridization of the DNA to an oligonucleotide probe that has been covalently attached to an oxidized porous ($0.4 \mu\text{m}$ diameter pore) poly(ethylene terephthalate) (PET) membrane⁸. An oligonucleotide that contains a 3' C-6 aliphatic amino-linker (which can be made by automated phosphoramidite synthesis) is covalently attached to an 8.5 mm oxidized PET membrane

via a water soluble carbodiimide reaction. The DNA to be detected can then be placed on the membrane, allowed to hybridize, and detected as an oxidative current enhancement in the cyclic voltammograms taken.

The research covered by this grant will require the further refinement of our electrochemical biosensor. Presently, the biosensor has only been used to detect PCR products and synthesized oligonucleotides, both of which are DNA chains that can be produced in large amounts. One of this research project's aims is to develop the sensor so that it will be able to detect specific rare mRNA sequences (namely RAK and Cdc2) from relatively small (10 μ g) RNA samples.

BODY

Experimental

Development of an Electrochemical Biosensor

A procedure similar to that of Napier et al. was followed with the intention of optimizing it for use in quantifying mRNA expression levels⁸. Briefly, a 7 mm poly(ethylene terephthalate) (PET) membrane (0.4 μm pore diameter) was oxidized for roughly 18 hours in a solution of 1.25 g KMnO_4 in concentrated H_2SO_4 . The oxidized membranes were washed twice in 6 M HCl to remove MnO_2 . For each experiment, the membranes were soaked for twenty minutes in an aqueous solution containing 20 mM N-ethyl-N'(3-(dimethyl)aminopropyl carbodiimide hydrochloride (EDC) and 5 mM N-hydroxysulfosuccinimide (NHSS). The EDC/NHSS soaking step was performed to activate the native carboxylates on the oxidized PET membrane. After washing, the activated membranes were placed in a constant humidity container on a glass sheet. 25 μL of a 100 μM solution of a probe oligonucleotide -- usually 20 bases in length -- was spotted on each side of the membrane and allowed to react with the membrane for a total of twenty minutes. The oligonucleotide used contained a 3' C-6 aliphatic aminolinker, and the probe solution was buffered to a pH of 9.0. We believe that the raised pH renders the aliphatic amine a strong enough nucleophile to attack the activated carbonyls on the membrane. Because the method of detection for our biosensor involves the oxidation of guanine, the probe sequences were not synthesized with guanine. If a guanine base was needed for the probe to provide complementarity to a certain mRNA, the probe was synthesized with the base hypoxanthine substituted for guanine. Hypoxanthine base pairs with cytosine through two hydrogen bonds, but it is not electrochemically active at the oxidation potentials required of our sensor.

Once the membranes were treated with probe sequences and washed, 10 μg aliquots of a total RNA sample in an 800 mM NaCl solution were applied to each membrane. It was assumed that the target mRNA of interest (i.e. the mRNA that contained

a sequence complementary to the probe sequence) would hybridize to the probe through Watson Crick base pairing. Hybridization was allowed to occur at room temperature for 1 hour in the constant humidity chamber. The hybridization temperature was kept low and the hybridization time kept short because of fear concerning exogenous RNase contamination.

After hybridization the membranes were again washed. Each membrane was then placed onto a cleaned indium tin-oxide (ITO) electrode, and the electrode was placed into an electrochemistry cell. A solution of 300 μM $\text{Ru}(\text{bpy})_3^{2+}$ in 50 mM sodium phosphate buffer (pH = 7.0) was added to the cell. The solution was allowed to diffuse through the membrane for 20 minutes. Following the 20 minute equilibration, cyclic voltammetry was performed over a potential range of 0-1300 mV vs. Ag/AgCl at a scan rate of 25 mV/s. The data were interpreted by comparing the peak currents obtained from sample to sample.

Quantification of RAK mRNA by S1 Nuclease Protection Assays

S1 nuclease protection assays were performed so that the actual expression of RAK mRNA could be quantified in a standard fashion. The assays were carried out according to the protocol supplied in the Ambion S1 Nuclease Protection Assay kit. Briefly, a 5' labeled oligonucleotide probe was added to a sample of total RNA containing the target sequence. The mixture was ethanol precipitated and redissolved in a proprietary buffer. The probe and target were allowed to hybridize for 18 hours. After the hybridization, S1 nuclease was added to the mixture. S1 nuclease cleaves all single stranded nucleic acid sequences so all non-hybridized probe and RNA chains were digested. The oligonucleotide probe was designed so that the last ten residues on its 3' end would not hybridize with the desired target sequence. This design served as a control to monitor if proper digestion of the probe by the S1 nuclease was taking place. The nuclease digestion was quenched, and the products were analyzed by gel electrophoresis (20% denaturing polyacrylamide). It was assumed that a band corresponding to ten bases shorter than the

length of the starting oligonucleotide would represent "protected" probe that had hybridized to the target RNA. This band's intensity could be compared to some standards, and the molar expression level of the gene of interest (RAK) could be determined.

Results and Discussion

Electrochemical Biosensor

A great deal of time was spent in trying to optimize the procedure for detecting rare mRNA's in cultured cell lines. The greatest challenge was trying to perform the procedure in such a way that the membranes would remain RNase free. A protocol was finally devised that involved minimal handling of the membranes from the time of oxidation until the time that they were analyzed electrochemically. However, this protocol was by no means a guarantee that the membranes remained RNase free, and we were not able to perform any experiments to show the level of RNase contamination on a given membrane.

Simple experiments using radiolabeled probe sequences revealed that 25 pmol (2.5×10^{-11} mol) (roughly 0.5% of the total amount added) of probe could be consistently attached to activated PET membranes. At the time these experiments were performed, it was believed that there were on the order of 100 amol (1×10^{-16} mol) of target sequences in the 10 μ g RNA samples that were being analyzed. So while the immobilization reaction used to attach the probe to the membrane was extremely inefficient, there was still a significant excess of probe immobilized to ensure the membrane would not be saturated by target strands.

Several different probe/target combinations were performed during the experiments. Probes for mouse β -actin were used with total mouse liver RNA samples. Probes for the FAK oncogene were used with RD cell (known to overexpress FAK) total RNA, and probes for the RAK oncogene were used with BT-474 cell (known to express RAK) total RNA. Because of the fears of RNase contamination, only three membranes were tested during any one experiment. In a typical experiment, one membrane contained probe only.

One membrane contained the probe plus some RNA sample that was not expected to contain a target that could hybridize with it (to test non-specific binding). And one contained the probe plus an RNA sample that contained the probe's target. Most of the original experiments showed an oxidative current enhancement that was greatest when the probe on the membrane was allowed to hybridize with the RNA sample that was known to

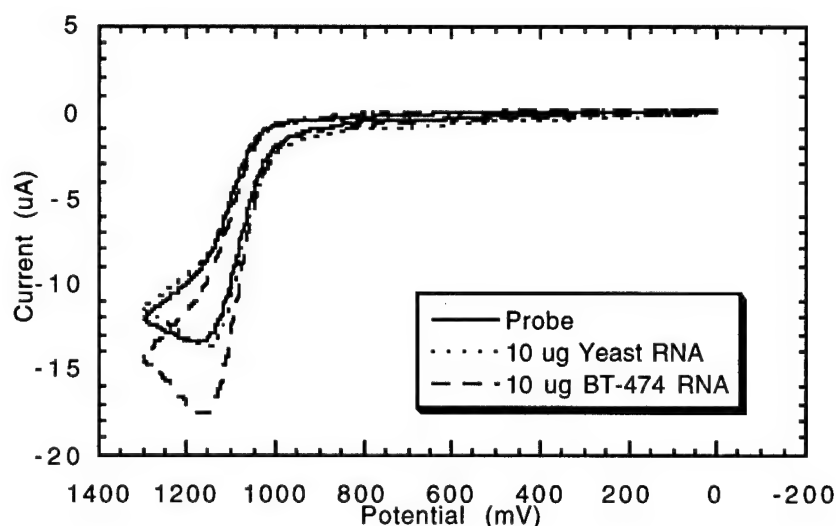


Figure 2: Representative data produced from the electrochemical biosensor. Three activated membranes had a probe complementary to RAK immobilized on them. One membrane then underwent cyclic voltammetry (line). One membrane had 10 μg of total yeast RNA added to it (dotted). And one membrane had 10 μg of total BT-474 RNA (which is known to express RAK) added to it (dashed). Similar data is available for FAK and β -actin mRNA, but because of concerns regarding the data, they are not presented.

contain the target. This was observed for all three targets (β -actin, FAK, and RAK).

While these results were very encouraging there are several concerns to be addressed. Competitive RT-PCR studies performed by Revillion et al. showed that HER-2 mRNA is expressed in cultured cell lines at levels of roughly 100 zmol (1×10^{-19} mol) per 1 μg total RNA⁹. While Revillion's data does not speculate of the abundance on RAK or FAK in cultured cell lines, HER-2 is a signaling tyrosine kinase like RAK and FAK, and it is reasonable to assume that RAK and FAK are also expressed at levels similar to those of

HER-2. The ramifications of these data for our study are that with such a small number of target molecules (and, consequently, available guanines for oxidation) the amount of electrons transferred from the target strand to the electrode would be expected to be very small. This small amount of electron transfer should, by a first approximation, yield current enhancements that are far smaller than the ones we observed.

Another concern is that the background current produced from membranes treated only with EDC and NHSS varied dramatically from experiment to experiment (and possibly from membrane to membrane). Unreacted EDC is readily oxidized at the potentials our experiments reach, and it has proven to be impossible to totally remove the unreacted EDC from the activated membranes despite vigorous washing steps. Because such a large amount of EDC was used to activate each membrane, current enhancements could simply be from variations in the amount of EDC adsorbed onto the membrane and not due to the actual amount of mRNA hybridized to the probe.

S1 Nuclease Protection Assays

S1 nuclease protection assays were chosen as the means to establish the actual mRNA expression level of RAK because they allowed the use of DNA probes to conduct the assay. Other nuclease protection assays involve using a single stranded RNase to cleave sample RNA and excess RNA probe. The advantage of using a DNA probe was that the probe could be synthesized and 5' labeled easily, while an RNA probe would have to be transcribed in some manner.

After performing several S1 nuclease protection assays, the problems with this technique became obvious. The major difficulty that we faced in this procedure was in generating a labeled probe sequence that had a high enough specific activity. 5' labeling of an oligonucleotide was only able to give us a specific activity of roughly 3×10^6 cpm per pmol. If the HER-2 expression levels from Revillion's research can be used as an approximation of that of RAK, any protection assay will have to use a probe that has a

much higher specific activity. This type of probe could be generated by producing a body labeled RNA probe through *in-vitro* transcription of an RT-PCR generated fragment of the mRNA of interest. Because of the low specific activity of the probe, it was not possible to determine the RAK expression level in BT-474 cells.

Recommendations

Since it came to our attention that the mRNA expression levels of RAK could potentially be very low, we have decided to work on alternate strategies to quantify RAK both through traditional and electrochemical techniques. We believe that competitive RT-PCR may be the best way to quantify RAK mRNA in total RNA samples because of its high sensitivity. Quantification of the PCR products could be performed both by agarose gel electrophoresis and by our electrochemical biosensor. To directly detect RAK mRNA electrochemically, it may be necessary to begin using microelectrodes with probes attached directly to them.

We recommend that we should continue working to quantify RAK expression in BT-474 cells first and then in tumor samples, albeit through different techniques than the ones employed previously. Competitive RT-PCR appears to be a suitable technique that would enable us to accurately quantify RAK in all RNA samples. We have recently begun RT-PCR experiments to try to amplify a segment of RAK mRNA. Once we have optimized the RT-PCR of the RAK mRNA, we will produce a competitor RNA sequence for use as a standard. We would then like to quantify the amount of PCR product by agarose gel electrophoresis. The results from the gel could then be compared to the results obtained electrochemically.

We believe that we could use our electrochemical approach to quantify the amount of RT-PCR product; however, we recommend modification of the strategy. We feel that we must modify our techniques because of the problems of EDC adsorption onto the PET membranes and the fact that the membranes had to be handled extensively. To address this

problem we have begun directly modifying the surface of the ITO electrode so that it can directly bind the probe DNA sequences. We are currently working with phosphonate self assembled mono-layers that contain a free carboxylate group on one end of each phosphonate molecule. The free carboxylate should make the mono-layer reactive to the primary amines on the oligonucleotide via our standard EDC/NHSS coupling reaction. Another approach we are pursuing is covalently attaching derivitized silanes onto the ITO surface, and then attaching the probe DNA sequence to the silane via a glutaraldehyde linking reaction. Both of these surface modifications do not affect the current response of $\text{Ru}(\text{bpy})_3^{2+}$ in solution, and both techniques have shown current enhancement in the presence of surface attached calf thymus DNA and guanine containing DNA oligonucleotides.

To develop an electrochemical technique whereby rare mRNA sequences can be detected directly, we recommend that we begin pursuing strategies to modify our biosensor so that it can be used with microelectrodes, which have a diameter of roughly $1\mu\text{m}$. Currently, ITO electrodes cannot be manufactured that small, so a different electrode material must be chosen. A good candidate for our purposes is glassy carbon. Glassy carbon electrodes can operate in aqueous solutions at potentials of 1300 mV, and they can be manufactured to sizes of $10\mu\text{m}$ in diameter. Although covalently attaching probe DNA to the glassy carbon surface does not seem to be feasible, it is possible to electropolymerize a film containing pyrrole-2-carboxylic acid directly onto the electrode surface. If the film is thin enough, $\text{Ru}(\text{bpy})_3$ should be able to diffuse through the film and reach the electrode surface. The carboxylates in the film would serve as sites where the probe DNA could be attached via the EDC/NHSS reaction. We have begun some electropolymerization experiments on 1.5 mm glassy carbon electrodes, and have been able to detect $\text{Ru}(\text{bpy})_3^{2+}$ at the electrode surface on electrodes that were coated with thin films of polypyrrole.

CONCLUSIONS

Electrochemical Detection of RAK mRNA

While the initial results obtained from our work with our biosensor indicated that we had detected (but by no means quantified) RAK mRNA (as well as FAK and β -actin), we cannot rule out the possibility that our results are simply due to random variations in the amount of EDC adsorbed onto the membrane or even inhomogeneities within the membranes themselves. The current enhancements we observed were larger than expected because RAK is likely expressed at low levels (comparable to those of HER-2). Because of the potential sources of error and the likelihood that RAK is expressed at a low level, we do not believe that our current method provides a viable approach for quantifying mRNA over the long term.

We hope to electrochemically detect RAK through either of two strategies. The first strategy is a more conventional approach in which we hope to produce an RT-PCR generated fragment of RAK and a competitor sequence and quantify the amount of DNA present electrochemically by hybridizing the products onto a surface modified ITO electrode. Our second strategy involves direct hybridization of RAK mRNA onto a polymer coated glassy carbon microelectrode that has an oligonucleotide probe attached to it. Work on both of these techniques is underway.

Quantification of RAK mRNA by S1 Nuclease Protection Assays

The S1 nuclease protection assay did not prove to be a sensitive enough technique to accurately quantify RAK expression. Nuclease protection assays could likely be used to quantify RAK expression; however, they would involve the use of a probe that has a much higher specific activity than the specific activity that is possible from a 5' labeled oligonucleotide probe.

To quantify RAK mRNA expression in a traditional manner, we believe that both competitive RT-PCR or ribonuclease protection assays that involve the use of a very high

specific activity RNA probe are viable methods. Because we are already performing RT-PCR for use in electrochemistry, we believe that competitive RT-PCR is the better option for us at this time.

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